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Synthesis of Lipids From Acetate-1-Carbon-14 by Leptospira Pomona.

Larry Ottis Arthur
Louisiana State University and Agricultural & Mechanical College

SYNTHESIS OF LIPIDS FROM ACETATE-1-^{14}C BY *LEPTOSPIRA POMONA*

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Larry O. Arthur
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Leptospira pomona was shown to incorporate radioactivity from acetate-1-^{14}C into extractable lipids. These lipids contained 67.7% of the total activity found in the cells. The majority of the radioactivity was associated with the point of sample application on chromatoplates used for neutral lipid and phospholipid separation. The triglyceride and sterol ester fractions were found to contain most of the radioactivity in the neutral lipid classes. Diglyceride, sterol, monoglyceride, and free fatty acid fractions were also labeled. Phospholipids contained 2 1/2 times as much radioactivity as did neutral lipids. The major phospholipid of L. pomona was found to be phosphatidyl ethanolamine. There were indications of phosphatidic acid and phosphatidyl glycerol. Phosphatidyl ethanolamine contained most of the radioactivity in the phospholipids, followed by phosphatidyl glycerol and phosphatidic acid. The glycerol-base portion of the phosphatidyl ethanolamine molecule, rather than the fatty acids, was thought to be labeled since the majority of the radioactivity was recovered from the water-wash after methylation. Fatty acids of the lipid classes were found to contain radioactivity with C 16:0 containing most of the activity from sterol esters, triglycerides, diglycerides, monoglycerides, and phosphatidyl ethanolamine. Stearic acid contained most of the activity found in the free fatty acid fraction. The ratio of radioactivity to mass was higher for C 15:0 than

other fatty acids, indicating more synthesis from acetate-1-^{14}C. A significant amount of activity was detected in fatty acids with 11 carbons or less.

CHAPTER I

INTRODUCTION

Leptospira pomona belongs to the order Spirochaetales and the family Treponemataceae. This organism consists of a spiral protoplasmic cylinder and an axial filament which runs the length of the cell inside the spiral and is connected to both ends of the cylinder. A membranous sheath encloses the cylinder and axial filament.

Some leptospira are pathogenic and produce a condition known as Weil's disease. This can range from a mild febrile reaction with no jaundice to profound icterus and death. The principle reservoir of the agent is wild rodents from which the organism is spread to domestic animals and man. Leptospiral infections are of great concern to the livestock industry due to decreased milk production, abortion and loss of weight.

Leptospira are obligate aerobes, and in some instances appear to be micro-aerophilic (Czekalowski, McLeod, and Rodican, 1953). They do not utilize carbohydrates and it appears that long-chain fatty acids serve as their sole carbon and energy sources. Most synthetic media employed for the cultivation of the organism have long-chain fatty acids present as carbon and energy sources.

Hexose, pentoses, deoxyhexoses, aminosaccharides, and hexosamines have been found in leptospiral cells

(Stiepanchonok-Rudnik, Potashova, and Chernukha, 1969). Parnas (1968) located 10 carbohydrates and 15 different amino acids in 14 strains of leptospira. Muramic acid and glucosamine have been found in the cell walls of L. icterohaemorrhagiae (Yanagawa and Faine, 1966). Hidalgo (1966), using thin-layer chromatography, located phospholipids, sterols, free fatty acids, triglycerides, and sterol esters in leptospiral extracts. He also reported that C 16:0, C 16:1, C 16:2, C 18:0, C 18:1, and C 18:2 accounted for an average of 80-92% of the total fatty acid composition of L. canicola, L. pomona, and L. icterohaemorrhagiae.

Radiotracer studies have revealed that radioactivity from oleate-1-^{14}C (Stalheim and Wilson, 1964a) and glucose-1-^{14}C (Ellinghausen, 1968) was incorporated into cellular material of L. canicola and L. pomona. Leptospira canicola has been reported to incorporate radioactivity from ^{14}C-acetate into non-lipid components, however, no activity was found in the fatty acids (Shenberg, Stern, and Tietz, 1966).

This investigation was undertaken in order to determine the extent of incorporation of acetate-1-^{14}C into lipids of L. pomona. Classes of lipids were separated by thin-layer chromatography and analyzed for radioactivity. Fatty acids from the various lipid classes were separated and identified by gas-liquid chromatography and the radioactivity of each of these fatty acids determined. The major phospholipids were separated and identified using two-dimensional thin-layer chromatography and analysis for

radioactivity on single-dimension plates.

CHAPTER II

SELECTED LITERATURE

A. GENERAL

The word Leptospira is formed by a combination of two Greek words leptus, meaning thin, and spira meaning spiral. This provides a very fitting description of the finely-coiled organisms which measure 6 to 20 microns in length and 0.1 to 0.2 microns in width. In liquid media either one or both ends are bent to form hooks.

Alston and Broom (1958) reported that the term Weil's disease was given to designate the form of infectious jaundice which Adolf Weil described in 1886. Broom (1952) stated that A. M. Stimson in 1907 was apparently the first person to see the causative agent of Weil's disease. Stimson found the organisms in sections of kidney from a patient who was believed to have died of yellow fever. He named the organism Spirochaeta interrogans.

The organism was again seen by Inada et al. (1916) in the liver tissue of a guinea pig injected with blood from a patient suffering from Weil's disease. They concluded that this organism was the causative agent of Weil's disease and named it Spirochaeta icterohaemorrhagiae. While working independently in 1915, Hubener and Reiter noted and described the causative agent as did Uhlenhuth and Fromme

(Broom, 1952).

Noguchi (1917) studied the Spirochaeta icterohaemorrhagiae of Inada and strains from Flanders, and considered them to be morphologically and immunologically identical. Noguchi (1918a) felt the organism should be renamed Leptospira because of the minute spirals running throughout the cell. He also observed that the organism did not have a flagellum. Unlike the other spirochetes, it resisted the destructive action of 10% saponin (1918b).

Since the creation of the genus Leptospira, many serogroups and serotypes have been discovered. The seventh edition of Bergey's Manual of Determinative Bacteriology (Breed, Murray, and Smith, 1957) lists 7 serogroups and 35 serotypes. The history and discovery of many of these serotypes were reviewed by Alston and Broom (1958).

Leptospires are found primarily in the kidneys of wild rodents and secondarily in the renal systems of domestic animals. They are shed in urine by an animal over a period of years and survive best in alkaline conditions in fresh water (Alston, 1961). Alexander (1970) pointed out they may survive 3 months or longer in neutral or slightly alkaline water. Gsell (1952) however, stated that leptospires do not survive long in water because of their relative sensitivity to cold, heat, dessication and above all, the action of other microorganisms.

There seems to be a range of pathogenic relationships from the relatively saprophytic state in rodents through the alternate phases of the disease and carrier state in

dogs and farm animals to the condition in man who seems to be highly susceptible but very seldom a carrier (Alston, 1961). These organisms are known to gain entrance to the body through intact mucous membranes and abraded skin and are believed to be capable of penetrating intact skin (Yager and Gochenour, 1960).

Leptospira are of tremendous economic importance, because they infect cattle and the result is fever, jaundice, abortion, loss of milk production, and loss of weight. The fact that they also cause disease in humans adds to their importance.

Clinical diagnosis of leptospiral infections is difficult during the first week or ten days. Direct dark-field examination is unsatisfactory due to the mistaken identification of fibrils and extrusions from red blood cells as spirochetes (Alexander, 1970).

During the first week of the disease the most reliable means of detecting leptospires is by direct culturing of blood in appropriate media. After the first week blood cultures are unsatisfactory, but urine samples may contain the organisms. Usually the samples are cultured directly in a medium containing 5-fluorouracil since this pyrimidine analogue does not inhibit leptospiral growth but may inhibit growth of contaminating organisms (Alexander, 1970).

Serological tests are also employed with the microscopic-agglutination test most often used. It is very sensitive and highly serotype-specific. Antibodies against

leptospires are usually present between the sixth and tenth days of the disease (Alston and Broom, 1958; Alexander, 1970).

Many antibiotics have been shown to inhibit leptospira *in vitro*. These include penicillin, chlortetracyline, streptomycin, oxytetracyline, erythromycin, Tropolon, and Hinokitiol (m-isopropyltropolon). Antibiotics, however, are ineffective against leptospira *in vivo* unless administered in the first three or four days of the illness. Therefore, these antibiotics seem to be of more value as prophylactic agents rather than for treatment of the active infection (Alston and Broom, 1958; Smadel, 1952).

Passive immunization seems to be effective as a prophylactic agent against leptospiral infections. Immunization of cattle against *L. pomona* and dogs against *L. canicola* and *L. icterohaemorrhagiae* is widely practiced. However, active immunization of humans against the organisms has been tried with little success (Alston and Broom, 1958).

B. NUTRITION OF LEPTOSPIRA

Leptospira were successfully cultivated *in vitro* by Inada et al. (1916) in an ascitic fluid-kidney medium formerly used for other spirochetes (Noguchi, 1912). Ellinghausen (1960) compared three commonly used media for growing leptospira (Schuffner's, Korthof's, and Stuart's) and found Stuart's to be superior. Cox and Larson (1957) described a solid medium which consists of tryptose-phosphate broth,

1% agar, rabbit serum, and a hemoglobin preparation. They reported obtaining surface colonies and stated that this would lend leptospires amenable to classical bacteriological techniques.

Schneiderman et al. (1951) found that much of the nutritive value of serum lies in the albumin fraction. In 1953 these workers reported that in this albumin medium, L. canicola growth was stimulated by arginine, aspartic acid, glutamic acid, or proline.

Johnson and Rogers (1964) found that a complete mixture of amino acids did not support growth of L. pomona. There is little asparaginase activity in L. pomona, but the ability to use asparagine as an ammonium ion replacement closely followed the asparaginase activity of the serum. Johnson and Gary (1962), using extensively dialyzed rabbit serum, found that L-asparagine was the only amino acid that markedly stimulated growth. Ammonium chloride and urea satisfactorily replaced L-asparagine and this indicated that amino acids served primarily as a nitrogen source.

Thiamine has been reported as an essential vitamin requirement for leptospiral growth (Schneiderman et al., 1953; Johnson and Gary, 1962). In addition, nicotinic acid, pantothenate, putrescine, p-hydroxybenzoic acid and vitamin B_{12} seemed to increase leptospiral growth (Vogel and Hutner, 1961). Stalheim (1964a) reported that biotin and vitamin B_{12} were essential for growth of L. canicola. Ellinghausen and McCullough (1965a) stated that since

rabbit serum is high in vitamin B_{12}, many previous workers who employed rabbit serum in their culture medium may have overlooked this vitamin as essential for leptospiral growth. Wooley and VanEseltine (1968) confirmed the absolute requirement for both thiamine and vitamin B_{12} by L. pomona.

Fulton and Spooner (1956) reported that minute amounts of some vital fatty acids were the only chemical changes noted in the media after growth of L. icterohaemorrhagiae. Helprin and Hiatt (1957) were first to study the effect of fatty acids on leptospiral growth. They found that fatty acids markedly stimulated the growth of L. icterohaemorrhagiae and the amount of stimulation was dependent on the length of the carbon chain.

Extraction of rabbit serum with chloroform reduced its growth-promoting activity for L. pomona, but addition of oleic acid or Tween 80 partially restored this activity (VanEseltine and Staples, 1961). Vogel and Hutner (1961) found that oleic acid esters would support growth of L. canicola.

Stalheim and Wilson (1964b) observed that L. canicola grew in a synthetic medium while L. pomona was rapidly lysed by the lipids in the medium. L. pomona did not grow in boiled serum. It was postulated that boiling destroyed the oleic acid-binding capacities of the serum. The formerly-bound fatty acids were subsequently freed and acted as lytic agents. These investigators (1964a) found that calcium, iron, magnesium, and ammonium ions, thiamine, and

a fatty acid source were the minimal components of a synthetic medium used to cultivate L. canicola. VanEseltine et al. (1967) revealed that addition of oleic acid or acetate to medium containing minimal serum concentrations resulted in both increased growth rate and cell crop. Shenberg (1967) devised a synthetic medium consisting of inorganic salts, EDTA, Tween 80 and 60, glycerol, L-asparagine, vitamin B_{12} and thiamine HCl. Ammonium chloride acted as the sole source of nitrogen. A fatty acid source, vitamin B_{12}, and ferrous ions were also essential.

Several attempts have been made at replacing the serum with other substances. Yanagihara and Mifuchi (1965) successfully substituted the growth-stimulating factor in rabbit serum with dead cells of Mycobacterium. Mycolic acid or its derivatives replaced rabbit serum for the growth of L. icterohaemorrhagiae (Azuma et al., 1968).

C. METABOLISM OF LEPTOSPIRA

Chang (1947) found that the growth of L. icterohaemorrhagiae conformed to a normal bacterial growth pattern except for the fact that each phase existed for a longer period of time. It had a generation time slightly less than two days at 23 C. The optimum growth temperature was 25-30 C. Ellinghausen (1960) determined that a temperature of 28.5 to 29.5 C and a pH of 7.4 were optimal for leptospiral growth. The minimal growth temperature for pathogenic leptospira is between 13 and 15 C (Johnson and Harris, 1968).

Growth was inhibited as the pH was lowered below 6.7 or raised above 7.8.

Leptospira exhibit a definite need for oxygen (Geiman, 1952). Czekalowski, McLeod, and Rodican (1953) considered leptospira to be microaerophilic. Fulton and Spooner (1956) demonstrated cytochrome c and Baseman and Cox (1969b) found evidence of cytochromes of the a, c, c_1, and o types. This, coupled with the fact that catalase has been demonstrated (Rao, Larson, and Cox, 1964) lends additional evidence that these organisms are aerobic.

Baseman and Cox (1969a) found that carbohydrate, short-chain substrates, or selected amino acids did not stimulate O_2 uptake as did Marshall (1949) when using various carbohydrates. Ellinghausen (1968) reported that glucose added to minimal media resulted in increased cell yield. Of sixteen carbohydrates employed, only glucose had a growth-stimulatory effect, and glucosamine and 2-deoxy-D-glucose inhibited growth.

Growth was not enhanced for L. pomona when purines, pyrimidines and nucleosides were added to the culture media. The spirochete is relatively insensitive to 5-fluorouracil indicating no direct incorporation of uracil into nucleic acids (Johnson and Rogers, 1964). VanEseltine et al. (1967) indicated that L. pomona did not exhibit requirements for purines or pyrimidines.

Intermediary metabolic studies of Baseman and Cox (1969a) revealed a respiratory quotient of 0.7 indicating the oxidation of lipid material. A comparison of Tween 80

and sodium oleate demonstrated that the long chain fatty acid of Tween 80 was the oxidizable substrate. Evidence showed that extensive metabolic lesions and not crypticity were responsible for lack of leptospiral growth on various substrates.

Jenkin et al. (1969) found that the 2- and 8- positional isomers of octadecenoic acid were not readily utilized by L. interrogans. However, the 3-, 4-, 6-, 11-, 15-, and 16- isomers stimulated growth similar to 9-octadecenoic acid (oleic acid). The 5-, 7-, 10-, 12-, 13-, 14-, 17- isomers resulted in an intermediate growth response. A combination of 9-octadecenoic and 2-octadecenoic acids increased the yield of the organism above the yield given with 9-octadecenoic acid. It was also noted that 400 ug/ml of 9-octadecenoic acid was inhibitory while 200 ug/ml of 9-octadecenoic acid plus 200 ug/ml of 2-octadecenoic acid was not inhibitory suggesting that two different enzyme systems were affected.

Green and Goldberg (1967), using gel electrophoresis, found esterase, acid phosphatase, and naphthylamidase activity in leptospiral extracts. Burton, Blender, and Goldberg (1970) confirmed the finding of naphthylamidase activity and stated that this activity was due to naphthylamidase and not aminopeptidase. Markovetz and Larson (1959) found evidence of transaminase in L. biflexa and Mifuchi, Hosoi, and Yamashito (1970) stated there was possibly an activator in rabbit serum for glutamic-pyruvic transaminase

activity in leptospiral cells. Lipase activity in leptospira has been well-documented (Patel, Goldberg, and Blender, 1964; Chorvath, 1968; Johnson and Harris, 1968; Berg et al., 1969; Chorvath and Fried, 1970). Evidence of oxidase (Goldberg and Armstrong, 1959) and catalase (Faine, 1960, Rao et al., 1964) has also been demonstrated. Baseman and Cox (1969a), using whole leptospiral cells, found succinate dehydrogenase, phosphotransacetylase, isocitrate dehydrogenase, NADH oxidase, citrate condensing enzyme, fumarase, and isocitrate, malate, succinate and α-keto-glutaric dehydrogenases. They found no kinase activity for substrates such as pyruvate, acetate, glycerol, ribose, and glucose and postulated that this could explain the apparent inability of leptospiral cells to utilize such substrates. Acyl dehydrogenase, a key enzyme in fatty acid oxidation, was also demonstrated.

D. BIOCHEMICAL COMPOSITION OF LEPTOSPIRA

Hiatt (1952) disintegrated L. icterohaemorrhagiae with sodium deoxycholate and found a soluble polysaccharide fraction which would fix complement. Schneider (1953) demonstrated pentose as well as deoxypentose nucleic acids. Stiepanchonok-Rudnik and Potashova (1969), using a fractionation technique described by Schneider (1954), reported the presence of hexoses, pentoses, methyl pentoses, deoxyhexoses, aminosaccharides, and hexosamines in leptospiral cells. Parnas (1968) studied 14 strains of leptospira and found arabinose, xylose, ribose, glucose, galactose, mannose,

rhamnose, abequose, glucosamine, and glucuronic acid. They also detected arginine, asparate, glutamate, glycine, histidine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tyrosine and valine. Yanagawa and Faine (1966) discovered muramic acid and glucosamine in the cell wall of L. icterohaemorrhagiae. Nauman, Holt, and Cox (1969) reported that an amino acid analysis of axial filaments from water strains of leptospira was similar in composition to amino acids of flagella from Spirillum serpens and Bacillus subtilis.

Stalheim (1968) observed that dried L. pomona cells yielded 12% lipid material. Allison and Stalheim (1964) reported L. canicola contained 16% lipid, of which approximately 50% was phospholipid. Octadecenoic acid comprised 47% of the fatty acids, followed by hexadecanoic (19%), and an unidentified, unsaturated fatty acid (9%) with a retention time slightly longer than pentadecanoic acid.

Livermore, Johnson, and Jenkin (1969) extracted the lipids from L. interrogans and found that 9-octadecenoic acid made up 37.6% of the fatty acids from the total lipid fraction, followed by hexadecenoic (26.3%), cis-11-hexadecenoic (22.3%), 9-hexadecenoic (3.0%), and octadecanoic (1.0%). The neutral lipid fraction also contained 9-octadecenoic (43.1%) as the major fatty acid followed by hexadecanoic (21.6%), cis-9-hexadecanoic (20.6%), 9-hexadecenoic (5.1%), and octadecanoic (2.8%). The phospholipids contained cis-11-hexadecenoic (28.9%), 9-octadecenoic (25.4%) and hexadecanoic (22.6%) as the major fatty acids.

Shenberg, Stern, and Tietz (1967) analyzed the effect of different fatty acids in the medium on the fatty acid composition of triglycerides and phospholipids. They concluded that L. canicola can select and incorporate the required fatty acid from the medium and desaturate stearic and palmitic acids to the corresponding monoenoic acids. Stern, Shenberg, and Tietz (1968) found that palmitate was dehydrogenated to 9- and 11-hexadecenoic acids, while stearate was only dehydrogenated to 9-octadecenoic acid. Using phospholipase A on phosphatidyl ethanolamine which was isolated from cells grown in Tween 60 and 80 media, they observed that oleic acid was in the α position and palmitic, stearic, and 11-hexadecenoic acids in the β position. Palmitate was shown to be in the β position and 9-hexadecenoic acid in the position. There was an even distribution of 11-hexadecenoic acid between the α and β positions of phosphatidyl ethanolamine isolated from L. canicola which was grown in media containing palmitate.

Hidalgo (1966) found phospholipids, sterols, free fatty acids, triglycerides, and sterol esters in L. canicola, L. pomona, and L. icterohaemorrhagiae grown in albumin-Tween 80 and Stuart's media supplemented with 10% rabbit serum. The most abundant fatty acids were C 16:0, C 16:1, C 16:2, C 18:0, C 18:1, and C 18:2 which accounted for 80% to 92% of the total fatty acids. He stated that the major fatty acids of L. canicola were strongly influenced by the fatty acids of the medium, indicating direct absorption of fatty acids from

the medium and incorporation into cellular lipids. The concentration of total extractable lipid of the three serotypes varied from 13.6% to 26.3%.

E. STUDIES INVOLVING LABELED PRECURSORS WITH LEPTOSPIRA

Stalheim and Wilson (1964a) reported L. canicola incorporated radioactivity from oleate-1-^{14}C into cellular components. The alcohol-ether fraction was found to contain 87.3% of the activity and the hot trichloroacetic acid fraction and protein residues contained 0.7% and 12.0%, respectively. The evolution of $^{14}CO_2$ from oleate-1-^{14}C indicated that L. canicola had fatty acid decarboxylase activity.

Radioactivity from glucose-U-^{14}C was incorporated into cell material (Ellinghausen, 1968). The alcohol-ether fraction contained the most activity followed by the hot trichloroacetic acid fraction, and the protein fraction. The cold trichloroacetic acid fraction contained very little radioactivity.

Johnson and Rogers (1967) revealed that L. pomona incorporated radioactivity from ^{14}C-8-azaguanine, adenine-8-^{14}C, and guanine-8-^{14}C into nucleic acids. The incorporation of radioactivity from palmitate-1-^{14}C into protein and DNA was used as an index of protein and DNA synthesis in the presence of purine analogues. Guanine reversed the inhibition of 8-azaguanine in L. pomona cells.

Labeled carbons from alanine, aspartate, glutamate, and glycine were used in the synthesis of proteins, lipids, and nucleic acids. Radioactivity from isoleucine, leucine, and valine were readily incorporated into proteins and would completely replace endogenously-synthesized amino acids (Johnson and Rogers, 1964). These investigators reported that radioactive guanine and adenine was found in the nucleic acids, whereas incorporation of radioactivity from labeled uracil, thiamine, or uridine was not noted in the nucleic acid fraction or in any other fraction. Radioactivity from ^{14}C-carbamyl aspartate was not found in L. pomona cells.

Shenberg, Stern, and Tietz (1966) reported that L. canicola grown in the presence of ^{14}C-palmitate would have radioactivity in both protein and lipid fractions; however, when grown in the presence of ^{14}C-acetate, the fatty acid moiety of the isolated lipids were devoid of radioactivity. Acetate was readily oxidized since radioactivity was found in non-lipid components of the cells.

III. MATERIALS AND METHODS

A. LEPTOSPIRA POMONA

The organism used in this investigation was the pomona strain of Leptospira pomona pomona. Cultures were obtained from the Veterinary Science Department of Louisiana State University. The organisms had been maintained for several years through weekly transfers in Stuart's rabbit serum medium. Stock cultures for this investigation were maintained in the same manner at 30 C.

B. MEDIA

The rabbit serum medium of Stuart (1946) was prepared by dissolving 3.4 gms of Bacto-Stuart's Medium Base (Difco Laboratories, Detroit) in 1000 ml glass-distilled water containing 5 gms glycerol and sufficient rabbit serum (Type I Hemolyzed, Pel-Freeze Biologicals, Inc., Rogers, Arkansas) to yield a final concentration of 10%. The pH was adjusted to 7.4 with sodium hydroxide prior to sterilization.

STERILIZATION. A Seitz filter, model 200/6, was equipped for double filtration with S-3 asbestos pads, and connected to five 4-liter Pyrex bottles by means of latex rubber tubing. The entire apparatus was sterilized by autoclaving for 1 hr at 121 C. Prior to filtration of the medium, the filter pads were washed by forcing 4-liters of

phosphate buffer, pH 7.4, through the filter assembly at a pressure of 2 to 4 psi. The medium was subsequently sterilized by filtration immediately after preparation at a pressure of 5 to 10 psi and collected in the five bottles. After completion, the tubing to each bottle was sealed with 2 screw clamps and cut distal to the clamps.

Tubing bells were attached to the side arm of the 4-liter flask by means of latex rubbing tubing. Medium for stock cultures was dispensed aseptically through the tubing bells into 20 X 125 mm sterile screw-cap tubes and one hundred ml of medium were similarly dispensed into sterile screw-cap milk dilution bottles to be used as inoculum. Sterility was checked by incubation at 30 C for 5-7 days prior to inoculation.

ISOTOPE. Sodium acetate-1-^{14}C was obtained from New England Nuclear Corp., Boston, Massachusetts and Amersham/Searle Corp., Arlington Heights, Illinois. The isotope was diluted in a minimal amount of distilled water, dispensed in 0.25 mc amounts in screw-cap tubes, and sterilized by autoclaving for 15 min at 121 C.

C. PROPAGATION AND HARVESTING

Cultures for inoculation of large volumes of medium were prepared by placing 5 ml of stock culture into 100 ml of medium in milk-dilution bottles. After incubation at 30 C for seven days the cultures were examined for purity by dark-field microscopy and 200 ml transferred into the

4-liter bottle of medium. The cells were incubated for four days at 30 C and 0.25 mc of sodium acetate-1-^{14}C was added and incubation was continued for three additional days at 30 C before harvesting.

Cultures were examined for purity by dark-field microscopy and killed by adding sufficient formalin to yield a final concentration of 0.5%. The formalin was allowed to react for 4-6 hrs at ambient temperature. Organisms were harvested by centrifugation at 16,000 X g using a GSA rotor in a Sorvall RC-2 refrigerated centrifuge at 4 C. The cells were washed three times in physiological saline at 27,000 X g, once in distilled water, suspended in five ml distilled water and immediately subjected to lipid extraction. Aliquots were removed and placed in tared aluminum foil moisture dishes and dried over night in a vacuum oven at 80 C for gravimetric analysis.

Flasks, equipped with a side-arm attachment, containing 250 ml of Stuart's rabbit serum medium were used to determine growth phases of the organisms. Turbidity was measured using a Coleman Nepho-Colorimeter Model 9 (Ellinghausen, 1959).

D. INCORPORATION OF RADIOACTIVITY INTO CELLS

Cells grown in the presence of acetate-1-^{14}C were harvested as previously described. The organisms were suspended in distilled water in a 5 ml volumetric flask and 0.1 ml aliquots were removed and placed in scintillation

vials. In order to decrease self-absorption of the beta particles, the leptospiral cells were solubilized by adding 0.6 ml of NCS solubilizer (Amersham/Searle Corp., Des Plaines, Illinois). This mixture was kept at ambient temperature for at least 2 hrs and 15 ml of a scintillation cocktail, (PPO and POPOP in toluene) were added.

E. EXTRACTION OF LIPIDS

All solvents for lipid extraction and thin-layer chromatography with the exception of diethyl ether were redistilled prior to use. Whatman #1 filter paper, used in filtering the extraction mixture, was defatted by refluxing in diethyl ether for 6-8 hrs in a Soxhlet apparatus.

A modification of the method of Folch, Lees, and Stanley (1957) was employed for lipid extraction and purification. Cells were suspended in a minimal amount of water, placed in at least 40 volumes of 2:1 chloroform-methanol (v/v) and stirred overnight at ambient temperature. The mixture was filtered through 4 thicknesses of "extracted" Whatman #1 filter paper which was washed three times with chloroform-methanol (2:1). The filtrate was concentrated to approximately 20 ml at 65 C under vacuum (Rotary Vacuum Evaporator, Wright Scientific Ltd., London, England) and added to sodium chloride moistened with chloroform in a separatory funnel. Approximately 200 ml of distilled water were added and the funnel shaken. Phases were allowed to separate, the chloroform layer removed and the aqueous phase

washed three times with chloroform. All chloroform phases were combined and dried over sodium sulfate for at least two hours at ambient temperature. The lipid extract was placed in conical shaped Pyrex tubes and stored under nitrogen at 4 C.

F. THIN-LAYER CHROMATOGRAPHY

PREPARATION OF PLATES. Standard (20 X 20 cm) Pyrex plates were washed with detergent, rinsed with tap water, and finally distilled water. Immediately before use the surface of the plates was scrubbed with Kimwipes moistened with methanol.

The stationary phase for neutral lipid separation was prepared by mixing 22.5 gms of Silica Gel G according to Stahl (E. Merck AG., Darmstadt, Germany) in 50 ml distilled water. The slurry was spread on the plates to a thickness of 0.25 mm using a Brinkman adjustable applicator. Plates were air-dried until a mat surface was observed and subsequently activated at 115 C for 30 min (Randerath, 1964).

For phospholipid analysis, plates were prepared by mixing 30 gms of silicic acid, without binder (Mallinckrodt Chemical Works, St. Louis), with 60 ml distilled water. The plates were prepared and activated similarly to plates for neutral lipid analysis.

SOLVENT SYSTEMS. Two systems for separating neutral lipids were employed. A mixture containing 250 ml of petroleum ether, diethyl ether, and 35 N formic acid,

84:15:1 (v/v) was prepared for neutral lipid separation. A single-dimension, double-development system described by Freeman and West (1966) was also employed for separating neutral lipids. It consisted of a polar solvent system of diethyl ether, benzene, ethanol, and acetic acid, 40:50:2:0.2 (v/v), and a nonpolar system of diethyl ether and hexane, 6:94 (v/v).

Phospholipids were normally separated using chloroform-methanol-water, 70:40:4 (v/v), as the solvent system (Renkonen and Varo, 1967). Acidic phospholipids were separated with a solvent system consisting of chloroform-methanol-acetic acid-water, 80:13:8:0.3 (v/v) (Skipski and Barclay, 1969). All systems were prepared fresh daily and mixed thoroughly for 3-5 minutes on a magnetic stirrer prior to use.

APPLICATION OF SAMPLES AND DEVELOPMENT. Plates were reactivated at 120 C for 30 min and placed in a desiccator in order to cool to ambient temperature prior to use. A lipid standard (Hormel Institute II-A) consisting of hydrogenated lecithin, oleic acid, triolein, cholesterol oleate, and cholesterol was applied to each plate. Purified monoolein and distearin were included with the Hormel Institute II-A standard when using the single-dimension double-development system. Phospholipid standards were lecithin, lysolecithin, sphingomyelin, phosphatidyl serine, phosphatidyl inositol, and phosphatidyl ethanolamine (Supelco, Inc., Bellefonte, Pennsylvania). A concentrated

solution of leptospiral lipid was applied in narrow band across the remainder of the plate, approximately 2 cm from the bottom.

The solvent mixture (250 ml) was placed in tanks lined with filter paper, and at least 30 min were allowed for equilibration before development. After the solvent front had reach 1 cm from the top, the plates were removed, dried under a stream of nitrogen or in a vacuum oven, and detection reagents applied.

DETECTION OF LIPIDS. Iodine vapor was used to detect fractions to be analyzed by liquid scintillation counting. Lipid classes used in fatty acid analyses were detected by spraying the plate with a 0.2% solution of 2', 7' dichlorofluorescein in 95% ethanol and observing for fluorescence under short and long wave ultraviolet light in a Chromato-Vue (Ultra-Violet Products, Inc., San Gabriel, California). Fractions were located, marked, and identified according to standards on each plate. Lipids were removed from the plates by scraping the silica gel which contained each class onto glassine weighing paper. Samples were placed into 30-ml glass-stoppered test tubes for transmethylation or in scintillation vials for liquid scintillation analysis.

Phospholipids were detected using the molybdenum reagent spray prepared as described by Dittmer and Lester (1964). The molybdenum, in an acid solution, reacts with phosphate esters yielding a blue-colored phosphomolybdate complex. Phospholipids containing amino groups were located by spraying the plates with a ninhydrin reagent spray,

heating at 110 C for 10 min, and observing for color development. Ninhydrin spray consisted of 3% ninhydrin (Pierce Chemical Co., Rockford, Illinois) in butanol containing 3% glacial acetic acid (Stahl, 1965).

TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY OF PHOSPHOLIPIDS. Leptospiral phospholipids were applied with a microliter syringe as a single spot at the right-lower corner of the plates. The first solvent system, chloroform-methanol-water, 65:25:4 (v/v), was allowed to ascend to 3 cm of the top of the plates. The chromatoplates were removed, dried in a vacuum oven, rotated clockwise 90 degrees and placed in the second solvent system. A mixture of diisobutyl ketone (2,6-dimethyl-4-heptanone)-acetic acid-water 80:50:10, (v/v), was used as the second solvent system. After the solvent front reached 1 cm of the top of the plate, the chromatoplates were removed, dried in a vacuum oven, and detection reagents were applied.

ZONAL SCRAPING. Leptospiral lipids were spotted in a thin 5 cm band in the center of a thin-layer plate. On each side of the band, two reference spots were applied to the chromatoplate. One reference spot consisted of leptospiral lipids, and the other contained the monoolein and distearin in addition to the Hormel Institute II-A standard. Plates were allowed to develop in the single-dimension double-development system as previously described. The chromatoplates were dried, the leptospiral lipid band covered, and the reference spots sprayed with 2', 7'-dichlorofluorescein. The entire plate was marked off in 1/2 cm

bands by drawing a 27-guage needle across the silica gel. Each of these 40 bands was scraped into a scintillation vial. The bands corresponding to the reference spots were noted and recorded. Plates containing separated phospholipids were similarly scraped.

G. SEPARATION OF PHOSPHOLIPIDS FROM NEUTRAL LIPIDS

Lipid extracts of leptospiral organisms were applied on a thin-layer plate and developed as described under Section F topic Zonal Scraping. The phospholipids which remained at the origin were removed by scraping the silica gel from the plate into glass columns (10 mm O:D) and eluted by forcing chloroform-methanol (1:2) through the column. The mixture was concentrated at 65 C under vacuum, and stored under nitrogen at 4 C.

H. LIQUID SCINTILLATION COUNTING

INSTRUMENT. Liquid scintillation analyses were conducted using a soft-beta counting Beckman Liquid Scintillation System equipped with a Teletype Model 33 typewriter, digital subtraction of background and preset error control. The instrument automatically employed a channel ratio method, which read out in "S" values, for quench correction. This external standard (^{137}Cs) attachment was employed in calculation of the quench correction curve and to correct for quenching. A background was taken before each experiment, and the automatic background subtract was employed to correct for background.

SCINTILLATION COCKTAIL. A liquid scintillation mixture consisting of 0.3% 2,5-diphenyloxazole (PPO), 0.01% 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) in toluene (Chase and Rabinowitz, 1962) was used in a portion of this investigation. If samples contained silica gel, a methylcellosolve-BBOT cocktail was employed (Snyder, 1968). The stock solution contained 36 g 2,5-bis-[2-(5-tert-butylbenzoxazolyl)]-thiophene (BBOT), 720 g naphthalene, 700 ml methylcellosolve (ethylene glycol monomethyl ether) and 1650 ml toluene. The working solution was prepared by mixing 1000 ml of the stock solution with 1250 ml toluene, 1000 ml methylcellosolve, and 108 ml distilled water. All fluors (PPO, POPOP, and BBOT) were of scintillation grade (Packard Instrument Company, Inc., Downers Grove, Illinois).

QUENCH CORRECTION. A quench correction curve was prepared as described by Wang and Willis (1965) by adding sufficient ^{14}C-toluene (Packard Instrument Company, Inc., Downers Grove, Illinois) to the methylcellosolve BBOT cocktail to yield 20 ul of ^{14}C-toluene/15 ml BBOT cocktail. Exactly 15 ml of this mixture was pipetted into scintillation vials and varying amounts of chloroform added to act as chemical quencher. Efficiency of the liquid scintillation counter was calculated using a sealed internal standard containing 113,000 disintegrations per minute and found to be 91.4%. The standard quench correction curve was constructed by plotting $\log_{10}$ "S" values vs. percent efficiency (Fig. 1).

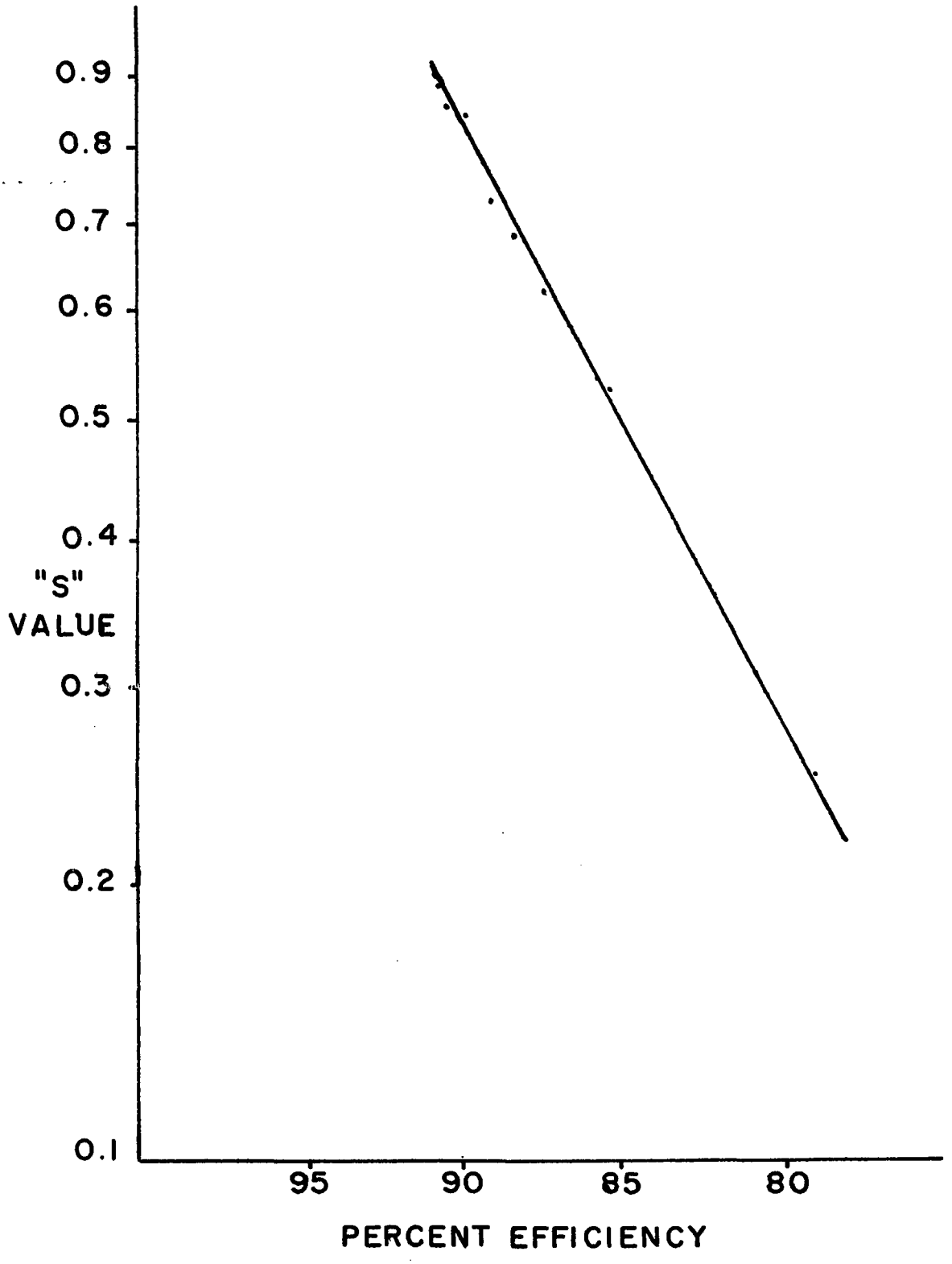


Fig. 1. Quench correction curve obtained by plotting $\log_{10}$ "S" values from the Beckman Liquid Scintillation System vs. percent efficiency of the instrument.

I. ESTERIFICATION

Fatty acids were prepared for analysis by gas-liquid chromatography by transmethylation procedure of Stoffel, Chu, and Ahrens (1959) as modified by Connellan and Masters (1965). The lipids to be transmethylated were placed in 30 ml glass-stoppered tubes with 5 ml of methylation mixture (dry methanol-benzene-36 N sulfuric acid, 20:10:1, v/v) and refluxed for at least 1 1/2 hrs at 130-165 C. The esters were extracted with 2 ml quantities of petroleum ether, washed three times with distilled water and placed over sodium sulfate for at least 1 1/2 hrs to dry. They were subsequently stored in conical shaped Pyrex tubes under nitrogen at 4 C. Prior to analysis in the gas-liquid chromatograph, the methyl ester solution was concentrated by placing the tubes in a water-bath at 60 C in a stream of nitrogen.

J. GAS-LIQUID CHROMATOGRAPHY

INSTRUMENT. Fatty acid analysis was conducted using a radiochemical gas chromatograph as designed by James and Piper (1961). It was equipped with a thermal conductivity cell as the mass detector and radioactivity was detected using a flow-through proportional counter. The column was connected directly to a combustion tube containing copper oxide and iron filings which was heated to 600 C. All organic material being eluted from the column was converted to CO_2 and water by the copper oxide, and the water was

reduced to hydrogen by the iron filings. Under these conditions, the thermal conductivity apparatus is very sensitive to hydrogen in the carrier gas (James and Piper, 1961; James and Piper, 1963).

The column temperature was maintained at 190 C. Argon flowed through the column at 50 ml/min and the flow rate was checked prior to sample analysis. The quencher gas, CO_2, was set at a flow rate of 6 ml/min.

COLUMN. A column made from copper tubing, 6 1/2 feet long and 1/4 in (O.D.) was washed with chlorform, dried, and packed with 20% polyethylene glycol adipate (EGA) on Chromport AW (80-100 mesh). EGA was prepared according to the method of James (1960). The column was bent to a configuration which would fit the oven and conditioned overnight at 210 C with an argon flow rate of 50 ml/min before being connected to the combustion tube.

IDENTIFICATION AND QUANTITATION OF FATTY ACIDS. A standard mixture of fatty acid methyl esters was chromatographed each day under the same conditions as the unknown samples. The standard contained saturated fatty acids from C 11:0 to C 18:0 and unsaturated fatty acids C 18:1 and C 18:2. A standard for radiochemical work was prepared by transmethylating 0.05 mc of palmitate-1-^{14}C (New England Nuclear Corp., Boston, Massachusetts) as previously described. Fatty acids were identified by comparison of observed retention time of unknowns to those of the standards. The area of the peaks was determined by triangulation (James, 1960).

K. TRAPPING $^{14}CO_2$ FROM THE GAS-LIQUID CHROMATOGRAPH

In order to increase the sensitivity of the radiochemical assay of the fatty acids, a procedure was devised in which the $^{14}CO_2$ was trapped and radioactivity determined using liquid scintillation counting. Woeller (1960) noted that using phenethylamine to trap $^{14}CO_2$ had certain advantages over other compounds used to bind $^{14}CO_2$. It was soluble in a mixture in which toluene was the principle component. It caused very little pulse quenching and the reaction with CO_2 was rapid and quantitative.

The phenethylamine solution, which was prepared fresh daily, contained 27% methanol, 0.5% PPO, 0.01% POPOP, 27% phenethylamine (2-phenylethylamine), and 10% naphthalene in toluene. The alcohol was added to increase the solubility of the carbamate of phenethylamine. Since the liquid scintillation counter operated at room temperature, naphthalene could be used which improved the counting efficiency. Increasing the amount of phenethylamine up to 35% increased the trapping efficiency of the solution.

Two ml of the phenethylamine solution was placed in 10 X 75 mm test tubes. The proportional counter of the radiochemical gas chromatograph was removed and a 30 cm long, 1/8 in (I.D.) silicone rubber tubing was attached to the outlet port of the thermal conductivity detector. The gas mixture (argon, carbon dioxide, and hydrogen) was bubbled through the phenethylamine solution by means of glass capillary tubing. Each peak, detected on the mass

recorder, was trapped in a tube. Immediately after the $^{14}CO_2$ from a peak was captured, the capillary was broken into the tube and the contents emptied into a scintillation vial. Ten ml of the scintillation cocktail were placed into each vial before counting in the liquid scintillation counter.

The efficiency of this technique for collecting $^{14}CO_2$ was determined by pipetting exactly 5 ul of the methyl ester of palmitate-1-^{14}C into a scintillation vial and placing 5 ul of this same methyl ester in the radiochemical gas-liquid chromatograph and trapping the $^{14}CO_2$. The counts of the former were compared to the counts of the latter and efficiency determined each day that samples were analyzed. Efficiency usually ran between 54% and 72% depending on the amount of phenethylamine in the solution. A background was determined by bubbling argon through 2 ml of phenethylamine solution for 15 min. This was converted to background per inch of chart since the chart speed was 15 in/hr. Background was corrected by determining the length of chart, which had unrolled for each captured peak, converting this to counts per inch using the value obtained for background, and subtracting this value from the counts obtained for the peak.

L. QUANTITATION OF NEUTRAL LIPIDS

Quantitation of neutral lipids was accomplished using the method of Amenta (1964). The principle behind this procedure was to measure, spectrophotometrically, the amount of acid dichromate which was reduced upon oxidation of

lipid material. The acid dichromate solution was prepared by dissolving 1.25 g of $K_2Cr_2O_7$ in 500 ml of 36 N H_2SO_4 and storing in a brown glass-stoppered bottle.

A thin-layer plate was divided into a number of lanes. Leptospiral lipid extract was applied to the center lanes, and the outer lanes contained standard samples. Classes of lipids were located by covering the lanes containing the leptospiral lipids and spraying the standards with 2', 7' dichlorofluorescein. The classes were scraped into 30 ml glass-stoppered test tubes containing 1 ml of acid dichromate solution. A silica gel blank equal to those bearing lipid was also placed in 1 ml of acid dichromate. The stoppered tubes were shaken and placed in a water-bath a 100 C for 45 min. During the heating period the tubes were shaken three times in order to disperse the silica gel. If the reduction of dichromate proceeded to a point that the contents were colorless, additional dichromate was placed in the tube containing the sample and in the tube containing the silica gel blank. Heating in the water-bath was then repeated. The silica gel was sedimented by centrifugation and 1 ml of the reduced acid dichromate solution was placed with 15 ml of distilled water. The optical density of the sample and blank were determined on a Beckman DB Spectrophotometer at a wavelength of 350 mu using an equimolar concentration of $KCr(SO_4)_2$ as a blank. Change in optical density was determined by subtracting the optical density of the sample from the optical density of the silica gel

blank. The quantity of lipid was determined by comparing the change in optical density to a standard curve constructed with the same amount of acid dichromate as used for the sample. Cholesterol and oleic acid were used as the standards and the change in optical density was plotted against µg lipid.

CHAPTER IV

RESULTS AND DISCUSSION

A. INTRODUCTION

Metabolic and biochemical investigations of the genus Leptospira have been limited, because leptospiral cell yields are very low, an enriched medium is usually required for growth, and there is a potential hazard due to their pathogenicity. Nevertheless, research on this genus indicates that the organisms utilize long-chain fatty acids as their carbon and energy sources.

Helprin and Hiatt (1957) were first to show that fatty acids stimulated the growth of L. icterohaemorrhagiae. Investigations on L. pomona (VanEseltine and Staples, 1961) and L. canicola (Vogel and Hutner, 1961) revealed that oleic acid and oleic acid esters would support growth. Fatty acids as the sole carbon and energy sources in several synthetic media have been reported (Stalheim and Wilson, 1964a; Ellinghausen and McCullough, 1965a; Shenberg, 1967). Therefore, it seemed quite likely that acetate, probably as acetyl CoA, was the building block of cellular material. In fact, VanEseltine et al. (1967) revealed that acetate added to a medium containing minimal serum concentrations resulted in both increased growth rate and cell crop.

However, questions have arisen as to the capability of leptospiral cells to synthesize lipids from acetate. Hidalgo

(1966) reported that the fatty acid profile of L. canicola was strongly influenced by the fatty acids in the medium. In fact, a suggestion was made that the organism preferentially utilized certain fatty acids of the various lipid classes. Shenberg et al. (1967), studied the effect of fatty acids in the medium on the composition of the fatty acids found in L. canicola. These workers believed that the organism selected the required fatty acids from the medium and desaturated them as needed. They also stated that ^{14}C-acetate was readily oxidized and radioactivity was found in cellular components; however lipids were devoid of radioactivity (Shenberg et al., 1966).

This investigation was undertaken to determine if L. pomona could utilize carbon atoms from acetate in synthesizing lipids. The amount of radioactivity from acetate-1-^{14}C incorporated into the neutral lipids, phospholipids, and fatty acids of these classes was determined. In addition, a phospholipid profile was determined for L. pomona.

B. RADIOACTIVITY OF CELLS AND LIPIDS

Nephelometric analysis revealed that all organisms harvested in this investigation were in the stationary phase of growth. Leptospira pomona, in this growth phase, yielded between 0.1 and 0.15 g of cells (wet weight) per liter of medium. Hidalgo (1966) reported obtaining 0.1 to 0.2 g (wet weight) of leptospiral cells per liter. Gravimetric examination indicated that approximately 20% of

L. pomona (dry weight) was composed of extractable lipids. This is slightly higher than than reported by Hidalgo (1966) who found 18% extractable lipids when L. pomona was grown in Stuart's serum supplemented medium. It is also higher than the 16% reported by Allison and Stalheim (1964) for L. canicola grown in a chemically-defined medium. Stalheim (1968) found only 12% extractable lipids in L. pomona cells. However, difference in organisms, growth conditions, or extraction procedures could account for these variations. Lipid content of most bacteria range from 1 to 10% (O'Leary, 1962); nevertheless, some exceptions are Bordatella pertussis which contains 24% lipid and human strains of Mycobacterium tuberculosis which contain 19.8% lipid (Asselineau and Lederer, 1960).

In the present investigation cells grown in a medium containing 239 μc of acetate-1-^{14}C incorporated 4.18 μc (1.75%) into cellular material. Extractable lipids contained 2.83 μc which was 67.7% of the radioactivity found in the cells. Ellinghausen (1968) reported that 42.5% of the radioactivity found in L. pomona cells, grown in the presence of glucose-u-^{14}C, was found in the alcohol-ether fraction.

C. NEUTRAL LIPIDS

Lipid classes corresponding to sterol ester, triglyceride, free fatty acid, sterol, and phospholipid standards were located in extracts from L. pomona, using thin-layer chromatography. The majority of the radioactivity (98.4%)

was found at the origin (Table 1). This prompted an attempt to separate the monoglycerides and diglycerides from the phospholipids since all three fail to migrate using petroleum ether-diethyl ether-formic acid as the solvent system.

Employing the single-dimension double-development system of Freeman and West (1966), the monoglycerides and diglycerides were separated. Phospholipids, which remained as the origin, were removed for further analysis. The majority of the radioactivity found in the neutral lipids was located in the fractions corresponding to the triglyceride and sterol ester standards and contained 38.4% and 23.5%, respectively (Table 2). The fractions which paralleled the sterol and diglyceride standards contained 12.4% and 12.0%, respectively. Approximately 5% of the radioactivity was found in the fractions corresponding to the monoglyceride and free fatty acid standards.

Quantitation of neutral lipids by acid dichromate reduction revealed that 30.2% was found in the free fatty acid fraction (Table 3). This was not surprising since the organism was grown in serum-supplemented medium which had a high fatty acid content. The fact that leptospira contain lipases which cleave fatty acids from triglycerides (Johnson and Harris, 1968; Berg et al., 1969; Chorvath and Fried, 1970) could also account for the high concentration of free fatty acids. The fraction corresponding to the monoglyceride standard contained two distinct bands; however, analysis of the fatty acids of the monoglycerides (Table 7)

Table 1. Incorporation of radioactivity from acetate-1-^{14}C into lipid classes of Leptospira pomona.

Lipid Classes[a]	cpm	% Radioactivity
Origin[b]	5.95×10^5	98.5
Sterols	2.81×10^3	0.5
Free Fatty Acids	2.16×10^3	0.4
Triglycerides	2.03×10^3	0.3
Sterol Esters	1.87×10^3	0.3

[a]separated by thin-layer chromatography using petroleum ether-diethyl ether-formic acid (84:15:1) as the solvent system

[b]included monoglycerides, diglycerides, and phospholipids

Table 2. Incorporation of radioactivity from acetate-1-^{14}C into neutral lipid classes of Leptospira pomona.

Lipid Classes[a]	cpm	% Radioactivity[b]
Monoglycerides	2.42×10^2	5.0
Free Fatty Acids	2.80×10^2	5.7
Sterols	6.05×10^2	12.3
Diglycerides	5.90×10^2	12.0
Triglycerides	1.88×10^3	38.5
Sterol Esters	1.29×10^3	23.5

[a]separated by thin-layer chromatography using the single-dimension double development system of Freeman and West (1966)

[b]origin was removed for phospholipid analysis and was not included in the calculation of % radioactivity

Table 3. Percent of neutral lipids found in each lipid class of Leptospira pomona.

Lipid Class[a]	% Neutral Lipids[b]
Monoglycerides	21.0
Sterols	9.3
Free Fatty Acids	30.2
Diglycerides	11.7
Triglycerides	15.4
Sterol Esters	12.5

[a]separated by thin-layer chromatography using the single-dimension double development system of Freeman and West (1966)

[b]determined by the acid dichromate reduction procedure of Amenta (1964)

revealed that C 18:1 and C 16:1 predominated. Chain length of the fatty acids in the molecule has been shown to affect migration on a thin-layer chromatography plate.

Analysis of fractions obtained by zonal scraping of the thin-layer plate demonstrated the profile of radioactivity in the neutral lipids (Fig. 2). Slight amounts of radioactivity were recovered in fractions corresponding to the monoglyceride and free fatty acid standards, with progressively increasing activities in sterols, diglycerides, triglycerides, and sterol esters.

D. PHOSPHOLIPIDS

Silica Gel without a binder was found to give better separation of phospholipid standards than Silica Gel G. Separation of leptospiral phospohlipid samples yielded only one spot detectable by the molybdenum reagent spray. This phospholipid gave a positive reaction with the ninhydrin spray and corresponded to the phosphatidyl ethanolamine standard. Shenberg et al. (1966) reported that the major phospholipid of L. canicola was phosphatidyl ethanolamine. They located another fraction which was thought to be cardiolipin (diphosphatidyl glycerol).

Two-dimensional thin-layer chromatography of the phospholipids of L. pomona (Fig. 3) revealed the presence of phosphatidyl ethanolamine. A very faint molybdenum positive spot was noted which migrated approximately the same distance as did a phosphatidic acid standard.

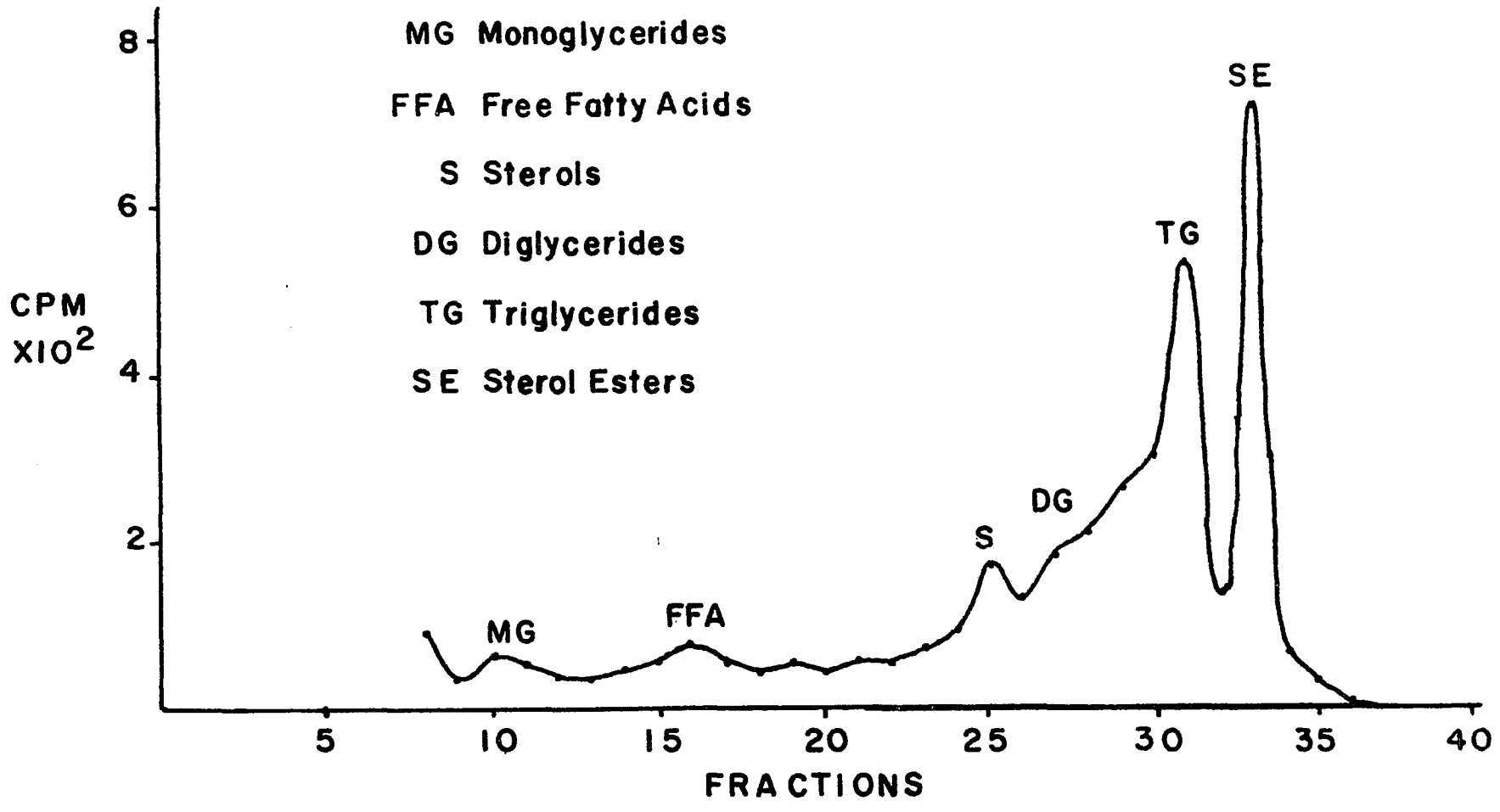


Fig. 2. Profile of radioactivity from a thin-layer chromatoplate on which neutral lipids of L. pomona were separated using the solvent system of Freeman and West (1966). Fractions 1-7 were removed for phospholipid analysis.

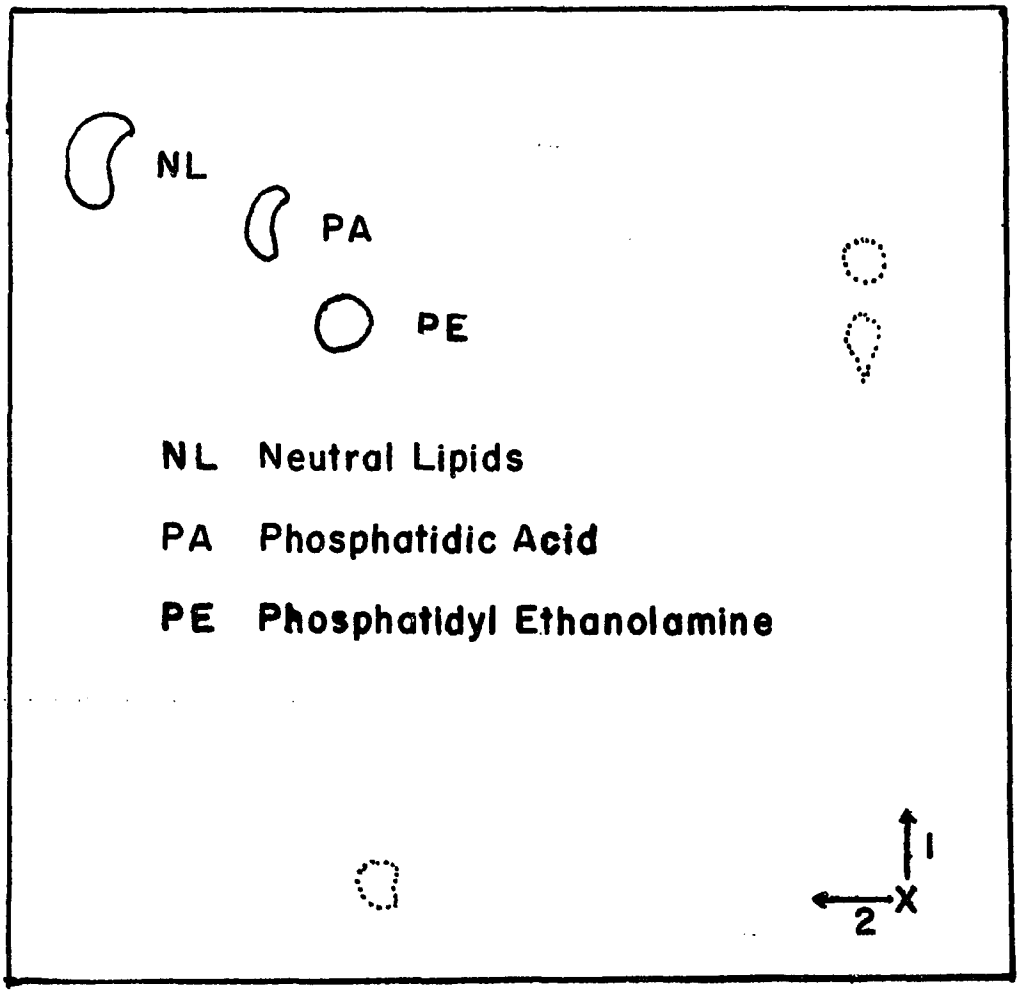


Fig. 3. Diagrammatic representation of a two-dimensional thin-layer chromatoplate of L. pomona phospholipids, developed in the first dimension with chloroform-methanol-water, 65:25:4 (v/v), and diisobutyl ketone acetic acid-water, 80:50:10 (v/v), in the second dimension.

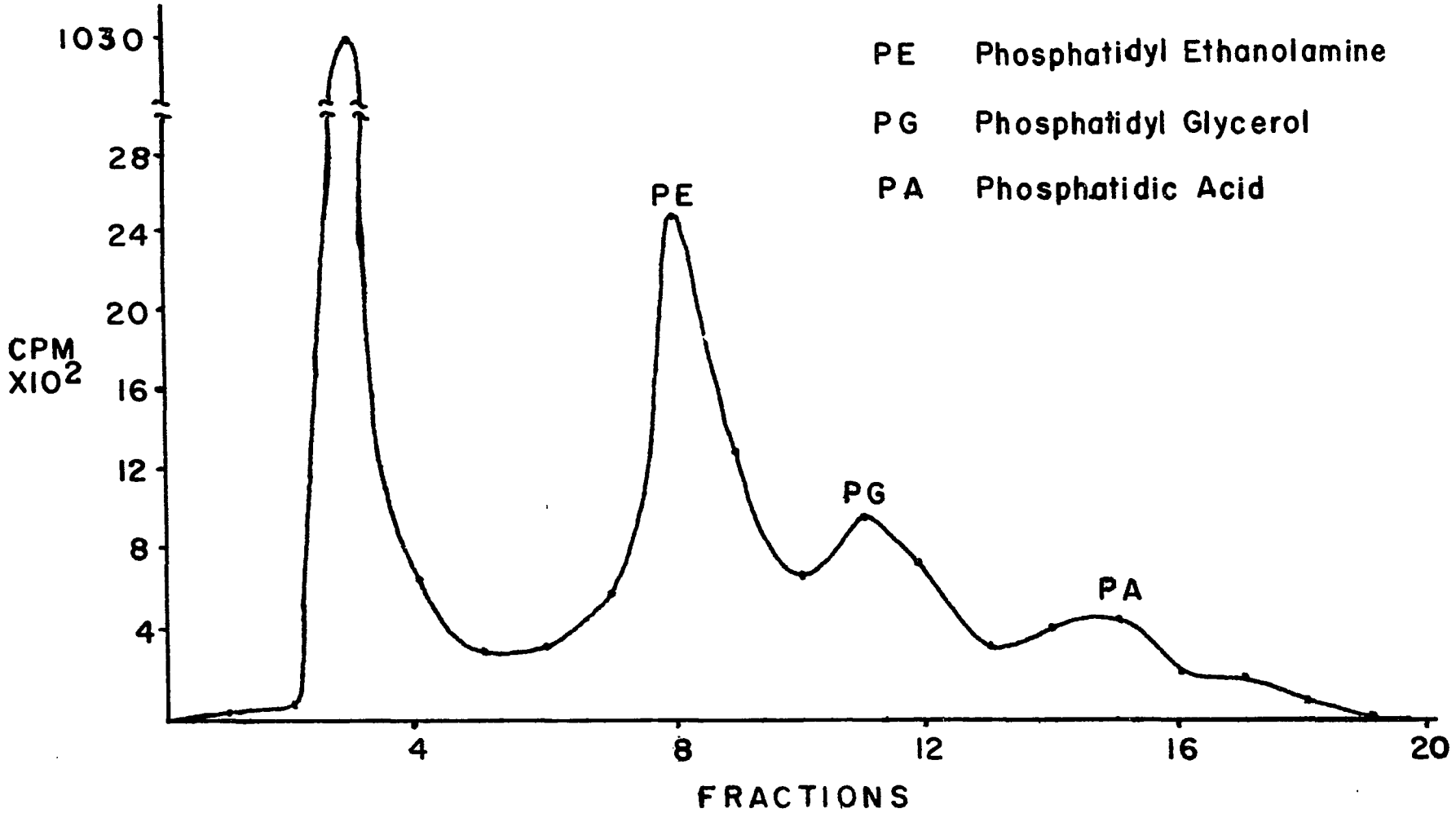
Positive phosphomolybdate spots, which are outlined in dots, were extremely faint and did not correspond to any standards.

Phospholipids were obtained by scraping the origin from a plate used to separate neutral lipids. This fraction was rechromatographed using a phospholipid solvent system and was found to contain 2 1/2 times as much radioacitvity as was present in the neutral lipids. Phosphatidyl ethanolamine had 62.4% of the radioactivity found in the phospholipids (Table 4). A fraction having a similar migration to that reported for phosphatidyl glycerol (Skipski and Barclay, 1969) contained 26.3% of the radioactivity followed by a fraction corresponding to a phosphatidic acid standard with 11.2%. As shown by zonal scraping, the majority of the radioactivity found on a phospholipid plate was located at the point of sample application (Fig. 4). Freeman and West (1966) reported that phospholipids and any highly polar material would remain at the origin on plates used to separate neutral lipids. Therefore, this polar material was removed from the plate and phospholipids separated by further thin-layer chromatography. Shenberg et al. (1966) reported a slow moving fraction which contained glycerol, inositol, and sugars on plates employed to separate phospholipids of L. canicola. Chemical identification was not attempted on this slow moving fraction which contained 93.4% of the radioactivity in this investigation.

Table 4. Distribution of radioactivity from acetate-1-^{14}C into phospholipids from Leptospira pomona.

Phospholipid Classes	cpm	% Radioactivity
Phosphatidyl Ethanolamine	7.78×10^3	62.4
Phosphatidyl Glycerol[a]	3.28×10^3	26.3
Phosphatidic Acid	1.40×10^3	11.2

[a]corresponded to reported Rf value, no standard was available to verify this tentative identification



Fig, 4. Profile of radioactivity on a thin-layer chromatoplate on which phospholipids of L. pomona were separated using chloroform-methanol-acetic acid-water, 80:13:8:0.3 (v/v).

After methylation of phosphatidyl ethanolamine, 94.6% of the radioactivity was recovered from the water-wash while only 5.4% was found in the petroleum ether phase which contained the methylated fatty acids. This indicated that the water soluble glycerol-base portion of the molecule was more extensively labeled than the fatty acid portion.

E. FATTY ACIDS

Methylated fatty acids were analyzed for radioactivity using a radiochemical gas-liquid chromatograph equipped with a proportional counter. Activity was not detected in the fatty acids of L. pomona. Using the methyl ester of palmitate-1-^{14}C, it was determined that radioactivity in a fatty acid would have to be approximately 15,000 cpm to give a detectable peak, therefore, the instrument was too insensitive to be used in analysis of radioactivity in lipids of L. pomona.

A more sensitive method of detecting radioactivity in fatty acids was devised in which phenethylamine was used to trap $^{14}CO_2$ from the gas-liquid chromatograph. The radioactivity in the phenethylamine carbamate was determined using liquid scintillation.

Over 24% of the radioactivity found in the fatty acids of the triglyceride fraction was associated with C 16:0 (Table 5) which also had the most mass area percent. There was a strong indication that short chain fatty acids were

Table 5. Incorporation of radioactivity from acetate-1-^{14}C into fatty acids of the triglyceride fraction from Leptospira pomona.

Fatty Acid	cpm[a]	% Radioactivity[b]	Mass Area%	Relative Radioactivity[c]
Below C 11:0	126.9	16.7	---	---
C 11:0	81.3	10.7	Tr[d]	---
C 12:0	36.4	4.8	0.2	---
C 13:0			3.54	1.4
C 14:0	66.9	8.8	1.77	5.0
C 15:0	42.4	5.5	0.6	9.2
C 16:0	184.8	24.35	38.16	0.6
C 16:1	60.7	8.0	3.41	2.4
C 17:0			0.68	
C 18:0	74.9	9.7	9.06	1.1
C 18:1	51.6	6.8	27.25	0.2
C 18:2	4.4	0.6	1.95	0.3

[a]counts per minute corrected for background from the gas-liquid chromatograph and $^{14}CO_2$ trapping efficiency

[b]percent of the total amount of radioactivity found in the fatty acids of the triglyceride fraction

[c]$\frac{\text{\% total radioactivity}}{\text{mass area \%}}$ = relative radioactivity

[d]trace

Percent error of cpm as calculated by the Beckman Liquid Scintillation System ranged between 3-10%.

labeled since significant radioactivity was collected before C 11:0 was detected. Stearic acid contained 9.7% of the total radioactivity of the fatty acids from the triglyceride fraction, while combinations of C 11:0 plus C 12:0 and C 16:1 plus C 17:0 yielded 10.7% and 9.7%, respectively. Relative radioactivity for C 14:0 and C 15:0 was high which suggested that these two acids were more actively synthesized from acetate-1-^{14}C than others of the triglyceride fraction. Only 6.8% of the total radioactivity was found in C 18:1 which contained 27.25% of the mass area. This indicated only minor synthesis of C 18:1 from acetate-1-^{14}C. The relative radioactivity of C 16:0 was low which indicated little synthesis of palmitic acid from acetate.

Fatty acids from the diglyceride fraction (Table 6) showed a pattern of radioactivity similar to the fatty acids from the triglyceride fraction in that most of the radioacitvity was recovered with C 16:0. A combined collection of C 16:1 and C 17:0 had 18.0% of the radioactivity. Compounds with 11 carbons or less contained 10.8%. Stearic acid contained 10.1% of the radioactivity followed by C 12:0 with 9.7% and C 15:0 with 8.9%. The relative radioactivity for C 15:0 was high indicating synthesis from acetate-1-^{14}C.

Radioactivity in fatty acids from the monoglyceride fraction (Table 7) differed from that found in fatty acids of the triglyceride and diglyceride fractions. Radioactivity was not detected in C 18:0 and C 18:1. However,

Table 6. Incorporation of radioactivity from acetate-1-^{14}C into fatty acids of the diglyceride fraction from Leptospira pomona.

Fatty Acid	cpm[a]	% Radioactivity[b]	Mass Area %	Relative Radioactivity[c]
Below C 11:0	95.2	10.8	---	---
C 12:0	85.6	9.7	Tr[d]	---
C 13:0	26.5	3.0	1.4	2.14
C 14:0	39.0	4.4	2.2	2.0
C 15:0	78.4	8.9	0.5	17.8
C 16:0	230.4	26.1	42.2	0.6
C 16:1	158.6	18.0	1.6	8.2
C 17:0			0.6	
C 18:0	88.9	10.1	12.5	0.8
C 18:1	70.3	8.0	27.5	0.3
C 18:2	10.3	1.2	11.4	0.1

[a]counts per minute corrected for background from the gas-liquid chromatograph and $^{14}CO_2$ trapping efficiency

[b]percent of the total amount of radioactivity found in the fatty acids of the diglyceride fraction

[c]$\frac{\%\text{ total radioactivity}}{\text{mass area }\%}$ = relative radioactivity

[d]trace

Percent error of cpm as calculated by the Beckman Liquid Scintillation System ranged between 3-10%.

Table 7. Incorporation of radioactivity from acetate-1-^{14}C into fatty acids of the monoglyceride fraction from Leptospira pomona.

Fatty Acid	cpm[a]	% Radioactivity[b]	Mass Area %	Relative Radioactivity[c]
Below C 11:0	99.1	12.5	---	---
C 12:0	77.6	9.8	---	---
C 13:0	5.9	---	Tr[d]	---
C 14:0	26.0	3.3	2.89	1.14
C 14:0, C15:0	162.8	20.5	0.90	22.8
C 16:0	225.9	28.5	43.3	0.66
C 16:1	187.2	23.6	---	---
C 18:0	0	---	17.7	---
C 18:1	0	---	35.2	---
C 18:2	8.7	---	Tr	---

[a]counts per minute corrected for background from the gas-liquid chromatograph and $^{14}CO_2$ trapping efficiency

[b]percent of the total amount of radioactivity found in the fatty acids of the monoglyceride fraction

[c]$\frac{\%\ \text{total radioactivity}}{\text{mass area \%}}$ = relative radioactivity

[d]trace

Percent error of cpm as calculated by the Beckman Liquid Scintillation System ranged between 3-7%.

C 16:0 was the most predominate both in radioactivity and mass area percent which was similar to the triglyceride and diglyceride fractions. Indications that C 14:1 and C 15:0 were largely synthesized from acetate-1-^{14}C were present because the relative radioactivity was high. Significant radioactivity was also found in C 16:1 and fatty acids with 11 carbons or less.

Fatty acids from the free fatty acid fraction (Table 8) were unique in that C 18:0 contained 37.5% of the radioactivity. Over 23% of the activity was associated with C 16:1 and C 17:0, while C 16:0 contained only 16% of the radioactivity. Significant synthesis from acetate-1-^{14}C was found in C 12:0, C 16:1, and C 17:0.

Significant activity was not detected in the fatty acids of the sterol ester fraction. It seems that the fatty acids esterified to the sterols were possibly selectively incorporated from the medium. This is an agreement with the hypothesis of Shenberg et al. (1967) that L. canicola can select required fatty acids from the medium and desaturate stearic and palmitic acids to the corresponding monoenoic acids. These workers deduced this by supplying various fatty acids in the culture medium and analyzing the fatty acids of triglycerides and phosphatidyl ethanolamine of L. canicola.

The majority of the radioactivity in fatty acids from phosphatidyl ethanolamine was associated with C 16:0 (Table 9). Over 20% of the radioactivity was associated

Table 8. Incorporation of radioactivity from acetate-1-^{14}C into fatty acids of the free fatty acid fraction from Leptospira pomona.

Fatty Acid	cpm[a]	% Radioactivity[b]	Mass Area %	Relative Radioactivity[c]
Below C 11:0	111.1	8.4	Tr[d]	---
C 12:0	41.1	3.1	0.3	9.4
C 13:0	35.3	2.7	---	---
C 14:0	46.3	3.5	1.7	2.12
C 14:1	15.1	1.14	Tr	1.3
C 15:0			0.9	
C 16:0	211.3	16.0	33.1	0.4
C 16:1	309.7	23.4	1.8	8.1
C 17:0			1.1	
C 18:0	496.7	37.5	30.0	1.25
C 18:1	56.7	4.3	22.1	0.2
C 18:2	---	---	9.12	---

[a]counts per minute corrected for background from the gas-liquid chromatograph and $^{14}CO_2$ trapping efficiency

[b]percent of the total amount of radioactivity found in the free fatty acid fraction

[c]$\frac{\text{\% total radioactivity}}{\text{mass area \%}}$ = relative radioactivity

[d]trace

Percent error of cpm as calculated by the Beckman Liquid Scintillation System ranged between 3-7%.

Table 9. Incorporation of radioactivity from acetate-1-^{14}C into fatty acids of phosphatidyl ethanolamine from Leptospira pomona.

Fatty Acids	cpm[a]	% Radioactivity[b]	Mass Area %	Relative Radioactivity[c]
Below C 11:0	60.4	5.5	---	---
C 12:0	12.5	1.1	Tr[d]	---
C 14:0	34.6	3.1	1.56	1.9
C 15:0	33.9	3.1	4.31	0.7
U[e]	29.9	2.7	Tr	---
C 16:0	353.7	32.3	34.4	0.9
C 16:1	93.4	8.5	8.5	1.0
C 17:0	27.7	2.5	1.1	2.3
C 18:0	149.0	13.7	4.4	3.1
C 18:1	220.7	20.1	33.4	0.6
C 18:2	82.8	7.5	12.3	0.6

[a]counts per minute corrected for background from the gas-liquid chromatograph and $^{14}CO_2$ trapping efficiency

[b]percent of the total amount of radioactivity found in the fatty acids of phosphatidyl ethanolamine

[c]$\frac{\text{\% total radioactivity}}{\text{mass area \%}}$ = relative radioactivity

[d]trace

[e]unknown

Percent error of cpm as calculated by the Beckman Liquid Scintillation System ranged between 3-7%.

with C 18:1. Stearic acid contained 13.7% of the activity and C 16:1 had 8.5%. All values of relative radioactivity were low with the exception of C 18:0 which was 3.1.

Shenberg et al. (1966) reported that no radioactivity was found in fatty acids of L. canicola grown in the presence of acetate-1-^{14}C. However, these investigators gave no experimental methods and an assessment of their data cannot be made.

Considering the small amount of radioactivity found in fatty acids of L. pomona, it is doubtful that the de novo synthesis of fatty acids from acetate is a major pathway. However, the fact that significant radioactivity is present in short chain fatty acids is suggestive that de novo synthesis from acetate does occur.

An interesting point in this investigation was the fact that the triglyceride fraction contained 38.5% of the radioactivity found in neutral lipids while only 5.0% was associated with the monoglyceride fraction. The monoglyceride fraction comprised 21.0% of the mass of the neutral lipids while the triglyceride fraction made up only 15.4%. The above factors suggest that fatty acids esterified to the glycerol contained the radioactivity. However, only small amounts of radioactivity, as compared to total radioactivity, were recovered with the fatty acids. A more appropriate explanation would be that perhaps acetate was esterified to the glycerol. The boiling point of the methyl ester of acetate is 57.1 C which means it would be lost in

the concentration procedure at 65 C. Esterification of acetate-1-^{14}C to the sterol could account for the high activity of the sterol ester fraction.

The relative radioactivity for C 15:0 was high in all neutral lipid classes indicating synthesis of C 15:0 from acetate. Palmitic acid contained most of the total radioactivity found in fatty acids of all lipid classes except the free fatty acid fraction. The majority of the radioactivity in the fatty acids of the free fatty acid fraction was found in C 18:0.

Hartman and Holmlund (1962) reported that bacteria can tightly bind the sterols present in a culture medium. Further investigation on L. pomona using labeled long-chain fatty acid would seem desirable to determine if the cells actually synthesize sterols.

This investigation has shown that L. pomona utilizes acetate-1-^{14}C in the synthesis of lipids. Radioactivity is found in neutral lipid classes with the majority of the activity in the glyceride and sterol ester fractions. The fatty acids of these lipid classes also contained radioactivity. The most predominant phospholipid in L. pomona is phosphatidyl ethanolamine.

CHAPTER V

SUMMARY AND CONCLUSIONS

An investigation was undertaken to determine the extent of incorporation of radioactivity from acetate-1-^{14}C into lipids of Leptospira pomona cultivated in Stuart's rabbit serum medium. Approximately 1.75% of the radioactivity from the medium was incorporated into L. pomona cells. Extractable lipids contained 67.7% of the radioactivity found in the organism.

Separation of neutral lipids by thin-layer chromatography revealed the majority of the radioactivity was associated with the triglyceride and sterol ester fractions, whereas, monoglycerides and free fatty acids contained the least. Other lipid classes containing activity were the diglyceride and sterol fractions. Phospholipids had 2 1/2 times as much radioactivity as did neutral lipids.

The major phospholipid of L. pomona was found to be phosphatidyl ethanolamine. Evidence of phosphatidic acid was found both by two-dimensional thin-layer chromatography and analysis of radioactivity on a single-dimension chromatoplate. Analysis of fractions obtained by zonal scraping suggested that phosphatidyl glycerol was present in the phospholipid extract of L. pomona.

Phosphatidyl ethanolamine contained most of the radioactivity found in the phospholipids followed by phosphatidyl

glycerol and phosphatidic acid. The majority of the activity found in phosphatidyl ethanolamine was recovered from the water-wash after methylation. This indicates that the glycerol-base portion of the molecule was labeled rather than the fatty acids.

The major portion of the radioactivity found in lipid extracts of L. pomona remained close to the point of sample application on plates used for neutral lipid and phospholipid separation. Attempts were not made to identify this material.

Fatty acids were analyzed by gas-liquid chromatography and the radioactivity determined by collecting the $^{14}CO_2$ in phenethylamine and subjecting this to analysis on the liquid scintillation counter. In all lipid classes, except the free fatty acid fraction, C 16:0 contained most of the activity. Stearic acid possessed the highest activity in the free fatty acid fraction. The relative radioactivity of C 15:0 was high in all neutral lipid classes indicating synthesis from acetate-1-^{14}C. A significant amount of activity was detected in fatty acids with 11 carbons or less.

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VITA

Larry Ottis Arthur was born March 14, 1944, in Many, Louisiana, the son of Mr. and Mrs. Ottis G. Arthur. He graduated from Florien High School in May of 1962 and entered Northwestern State College received a B.S. degree with a major in Microbiology and a minor in Chemistry in May 1966. He enrolled in Graduate School of Northwestern State College in September 1966 and received a M.S. degree in Microbiology in January 1968.

Work at LSU was initiated in January 1968 on a graduate training grant provided by the National Institutes of Health. He is presently a candidate for the degree of Doctor of Philosophy in Microbiology with a minor in Biochemistry.

EXAMINATION AND THESIS REPORT

Candidate: Larry O. Arthur

Major Field: Microbiology

Title of Thesis: Synthesis of Lipids from Acetate-1-^{14}C by Leptospira pomona.

Approved:

G. D. Dunnigan
Major Professor and Chairman

Max Goodrich
Dean of the Graduate School

EXAMINING COMMITTEE:

R L Allen

H D Braymer

M D Sokoloff

A. D. Larson

Date of Examination:

July 21, 1970